

EVALUATION OF AN ERYTHROPOIETIC FACTOR OBTAINED
FROM BLOOD OF EXPERIMENTALLY-INDUCED ANEMIC
CATTLE USING RADIOACTIVE Fe^{59}

by

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INTRODUCTION

In 1906 Carnot and Deflandre (15) described an erythropoietic stimulating activity of the serum of rabbits made anemic by bleeding. This finding created little interest for some 40 odd years. The humoral regulation of the erythropoietic phenomena was confirmed by European investigators. Erslev in 1953 (41, 42, 43); Borsook, et al. in 1954 (11); and Gordon, et al. in 1954 (75) reported definite findings that stimulated more research in the field of erythropoiesis in the United States.

Erythropoiesis has received a great deal of recognition since 1950 and including the present time. There are a multitude of publications contributing to the relatively new concept of an erythropoietic factor(s) or humoral agent(s) circulating in the blood stream. The new idea and the interest that erythropoiesis has brought about, inspired and stimulated many advancements and much of our knowledge of the mechanisms which regulate the production of red blood cells. The term "erythropoietin" is used to designate the factor(s) found in plasma of animals made anemic by bleeding or by other methods, which accelerates erythropoiesis when such plasma is injected into assay animals.

The erythropoietic stimulating activity of rabbit plasma and serum was described by Carnot and Deflandre in 1906 (15). They used the name "hemopoietine" for the unknown substance which stimulated red blood cell formation. Although the plasma factor's chemical nature and mode of action still are not completely understood, some of its physical characteristics have been

described. The name "erythropoietin" was suggested by Bonsdorff and Jalavisto in 1948 (10), and has been widely accepted by most investigators.

Most of the investigations on erythropoietin that have been accomplished utilized laboratory animals such as rabbits, hypophysectomized rats, and starved rats. Borsook (11) reported in his original work that the plasma of rabbits with phenylhydrazine-induced anemia had some erythropoietic activity. Some investigators (143, 144, 145) stated that this method was ineffective. Jacobson (90) first noted that boiled plasma from phenylhydrazine-treated animals did not produce a uniform response in erythropoiesis. Mirand (116) outlined a simple test for erythropoiesis in partially-deproteinized plasma extract enables a cross-species examination for the erythropoietic factor(s) in plasma.

PURPOSE OF STUDY

The following experiments herein reported were designed to:

- a. Demonstrate whether an erythropoietic factor(s) was present in bovine plasma;
- b. Assay the potency of bovine erythropoietin;
- c. Determine its cross-species potency and possible uses following an extended period of storage.

The data obtained would further the knowledge of the functions, availability, and uses of erythropoietin. Also, the data might verify the findings of other investigators' work on laboratory animals and apply this knowledge to cattle.

REVIEW OF LITERATURE

Factors Influencing Erythropoiesis

Oxygen. The primary function of the erythropoietic tissues is to produce hemoglobin-containing red blood cells capable of carrying oxygen from the lungs to the tissues. It has been known for some time that when the oxygen supply and consequently the tissue tension of oxygen are inadequate for cellular metabolism, a compensatory increase in red blood cell production occurs. Conversely, when the oxygen supply is too great and the oxygen tension in tissues is unnecessarily high, there is a decrease in red blood cell production. These facts have led to the conclusion that the tissue tension of oxygen controls the production of red blood cells in the bone marrow (29).

When the basic essential nutritional and endocrine requirements have been met, normal red blood cells are produced, each containing a specific amount of hemoglobin. The rate of production of these cells seems to depend primarily, if not altogether, on the degree of tissue oxygenation. Birkhill, et al. (7) demonstrated that when the circulating red cell mass was increased by 40 per cent, inducing a corresponding increase in tissue tension of oxygen, there was an almost complete cessation of red blood cell production. On the other hand, when the circulating red blood cell mass decreased, there was a direct proportional increase in red blood cell production until the erythrocyte cell mass and the erythropoietic function reached normal (44, 45).

Other workers contended that when the tissue tension of oxygen decreased below normal, there seemed again to be a linear relationship between the decrease in tissue tension of oxygen and the increase in red cell production (135). When the tissue tension of oxygen reached extremely low values, erythropoietic tissue suffered from the anoxia and the accelerated erythropoietic activity decreased (148).

The widely accepted theory, that the partial pressure of oxygen in the blood supplying the bone marrow represents the primary stimulus of erythropoiesis was based on the simultaneous findings of a low oxygen saturation in the arterial blood and an increased red cell production during hypoxia. Reissman, in 1950 (133), with the use of parabiotic rats (artificial Siamese twins), concluded that the stimulus was not the partial pressure of oxygen in the bone marrow directly but a humoral factor elicited by the hypoxemia in the one partner and transferred to the other mate. Recently it has been shown by Prentice and Mirand (126) that normal rats placed in a low oxygen atmosphere did not elaborate a factor in their plasma capable of stimulating increased incorporation of Fe^{59} into red cells of normal or hypophysectomized recipients.

Since the tissue tension of oxygen was not uniform throughout the body, it must be assumed that the red cell production was controlled by the tissue tension in a specific organ (44, 47). The tension of oxygen in this hypothetical regulatory erythropoietic center depended on its supply of oxygen and its rate of

oxygen consumption. If the supply of oxygen decreases, the tissue tension will be lowered, in some way causing an immediate compensatory increase in the mitotic rate of the erythropoietic tissue. Erslev (45) stated that tissue anoxia would have induced compensatory adjustments in the pulmonary and cardiovascular functions. These adjustments would not restore the tissue tension in the erythropoietic center to normal or otherwise the anoxic stimulus to red cell production would immediately be prevented or discontinued. However, it was possible that the erythropoietic center was located in an organ from which, during tissue anoxia, blood was shunted to vital oxygen sensitive tissues (45). This local diversion of blood away from the erythropoietic center would maintain a reduced oxygen tension in the center until final adjustments were accomplished by an increase in red cell volume (45).

This hypothesis of control of erythropoiesis by anoxia in bone marrow has diminished during the last few years. Erslev (47) observed and delineated that the anoxic stimulus operates in the bone marrow by accelerating the differentiation of stem cells into pronormoblasts, and that thereafter the maturation and multiplication of differentiated nucleated red blood cells proceed at fixed rates independent of the anoxic stimulus. Direct measurement of the oxygen tension of the bone marrow in acute and chronic anemia has failed to demonstrate a significant tissue anoxia (5, 77). In vitro studies by Magnussen (110) and Thomas (2, 150) of bone marrow revealed that the growth rate of erythroblastic cells and the heme production were depressed or unchanged rather

than stimulated by a low oxygen tension. Finally, the recent demonstration of a humoral erythropoietic factor made it unlikely that the erythropoietic function was regulated directly by changes in the tissue tension of oxygen in the bone marrow. The discovery of an erythropoietic factor led to the assumption that the erythropoietic center was located in an extramedullary organ or cellular system and that it controls red blood cell production by releasing a certain amount of an erythropoietic factor into the general circulation. This postulate or hypothesis has led many workers to investigate different substances, organs, and fluids of the body as the source or site of the mysterious erythropoietin.

Hormones of Endocrine System. The endocrine glands may exert an influence on the formed elements of the blood. In a classical paper, Aschner reported in 1912 (1) a decreased erythrocyte count and a decreased hemoglobin content of the blood of a hypophysectomized dog. Later it was shown that hypophysectomy induced an anemia in adult rats. The anemia could be prevented by the following therapies: thyroxin and androgen (31); thyroxin, cortisone, and growth hormone (113); thyroxine (25, 114, 115); combination of thyroxine, iron, and copper (24); androgen, either directly or induced by gonadotropic hormone (25, 162, 163); a combination of thyroxine, androgen, and a high protein diet (20, 31); testosterone propionate (25, 28, 32); adrenocorticotropic hormone (58); and cobalt nitrate (27), but growth hormone (28, 111, 113, 159) seemed to be ineffective.

Van Dyke and associates (160) found that anemia following hypophysectomy was more severe than the anemia following thyroidectomy, adrenalectomy, and gonadectomy alone or combined. They proposed that the "erythropoietic hormone" from the pituitary was the explanation for this finding. Van Dyke (161) also stated that the anemia which follows hypophysectomy apparently is due to the absence of the anterior lobe of the hypophysis as removal of intermediate and posterior lobes did not change the hemoglobin concentration, hematocrit, or volume of circulating red cells. Crafts (23) advanced the theory that large doses of estrogens exert a profound influence upon the cellular blood picture of some species of animals such as dogs and monkeys, since they developed hemorrhagic purpura, anemia, and leucocytosis followed by a leucopenia. Furthermore, Fruhman and Gordon (56) reported that growth hormone and cortisone were antagonistic to one another in their effects on development of both erythrocytes and white blood cells.

In 1938, Flaks, et al. (53) made an extract of cattle pituitary glands, fed it to hypophysectomized rats, and reported that erythropoiesis was stimulated. They claimed the presence of an erythropoietic "hormone" or "factor" in the anterior lobe of the hypophysis. Crafts (31) emphasized that evidence was gradually accumulating which indicates that the anemia which was induced by hypophysectomy was due to faulty metabolism and that there was no necessity for an "erythropoietic factor" or "hormone" secreted by the hypophysis. Contopoulos and co-workers (18)

reported they could not find this factor in cattle pituitary glands, but they did find it in sheep glands. Two additional reports followed the former allegations (20, 160), claiming the presence of a pituitary erythropoietic factor. In all cases the pituitary extract was administered orally. Crafts (28, 29, 30, 31) commented that it was strange that a pituitary hormone could be effective when given orally since other known hormones from the hypophysis are ineffective per orum.

Garcia (58) contended that of the known pituitary trophic hormones, only adrenocorticotrophically active preparations were able to repair the post-hypophysectomy anemia when injected in large doses into hypophysectomized animals. Thus, there is evidence supporting the concept that adrenocorticotrophic hormone acting through the adrenals is not the principal stimulating erythropoiesis, but that a contaminant in these preparations is responsible for the increased red cell production (20). The pituitary erythropoietic factor injected in small doses for short periods, inducing a polycythemia, appeared to support the concept of the importance of this substance in erythropoiesis. Its presence is necessary as a stimulant in red cell production, and in its absence interferes with the normal output of red cells by the bone marrow (20). Crafts (26) emphasized that it was safe to conclude the anemia induced by removal of the hypophysis was due to faulty metabolism and there was no necessity for a "hemopoietic hormone" secreted by the hypophysis.

Crafts (22) maintained in order to demonstrate the influence of the hypophysis on erythropoiesis it would have to be secondarily through the thyroid or the adrenal glands and three steps were necessary:

- a. Removal of either the thyroid or the adrenal glands should produce the same results as the removal of the hypophysis.
- b. The hormones produced by either the thyroid or the adrenal glands should prevent the symptoms caused by the removal of the hypophysis.
- c. A pituitary preparation should not be able to stimulate erythropoiesis in the absence of the thyroid or the adrenal glands.

Finch and his co-workers (51) maintained that the most striking and significant hematologic effect of adrenocorticotrophic hormone and cortisone was the acceleration of red blood cell formation and the increase in total circulating red cell mass. Fisher (52) discovered that adrenalectomy with cobalt gave negative results, while cobalt chloride and hydrocortisone increased the red cell volume, hematocrit, hemoglobin, and red blood cells. He also stated that the erythropoietic response to hydrocortisone and cobalt was greater together than either one alone. Blood volume determinations proved that these changes represent absolute increases in red cell mass and not hemoglobin concentration.

The most probable explanation of the erythropoietic action of adrenocorticotrophic hormone and cortisone appeared to be that by controlling the underlying inflammatory disease (rheumatoid arthritis, scleroderma, etc.), which release the bone marrow from the depressant effects of these diseases and allow a resumption of normal hematopoietic function. This was supported also by the fact that erythropoietic response occurred only in patients who showed improvement in their underlying disease. If adrenocorticotrophic hormone and/or cortisone therapy did not alleviate their arthritis, there was no improvement in their anemia. It should be emphasized that no pituitary extract has been prepared that will stimulate erythropoiesis which does not contain adrenocorticotrophic hormone, and no adrenocorticotrophic hormone has been found free of erythropoietic activity (157).

In conclusion, there is no doubt that the endocrines have a secondary influence on the essential nutrients for erythropoiesis through their influence on general metabolism. Whether they have a direct influence is still problematical (30). Crafts and Meineke (34) emphasized that the hypophysis has a profound influence on erythropoiesis which they believe to be secondary rather than primary.

Kidney. Jacobson (93) reported the inability of the nephrectomized rat to produce erythropoietin under a wide variety of conditions. He was not able to reproduce the observation of Mirand (117) who maintained that erythropoietin was still manufactured in nephrectomized animals in response to hypoxia, though

not in response to bleeding. Jacobson (93) postulated that the kidney was the site of production of erythropoietic factor(s) since bleeding and administration of cobalt did not produce circulating erythropoietin in the absence of the kidneys. Goldwasser and his co-workers (65) indicated that bilateral nephrectomy eliminated the normal capacity of rabbits and rats to elevate the circulating erythropoietin titer in response to the stimuli of anemia or the injection of cobaltous chloride. Bilateral ureteral ligation reduced but did not eliminate this capacity to respond, as Jacobson and his co-workers (93) found that by ligating both ureters they reduced but did not eliminate the capacity of the animals to produce an increased plasma level of erythropoietin. However, nephrectomy completely abolished the response (67).

It was suggested by Jacobson and colleagues (93), in their previous work, that the kidney was related to erythropoietin production. If this were correct, then the embryo must be able to derive it from an anlage, since erythropoiesis was initiated in the embryo before the kidney was established as a functional unit in the ordinary sense. Gallagher and his co-workers (57) stated that 15 of 16 human anemic uremic patients showed no active erythropoietic stimulating factor(s). If further work in this field makes quantitative measurement of erythropoietin activity possible, we will be in a much better position to determine whether the kidney is the sole producer of erythropoietin(s), is a co-producer, or is not involved at all, and that our

experimental approach has succeeded only in further clouding the situation (93).

Liver. The liver was alleged as a site of production of erythropoietin, but data now obscure this theory. A proposed hypothesis (90) was that the factor was produced in quantity only when the effective hematocrit was below 12 to 15 per cent and when the liver was not capable of rapid active destruction of the factor or was not producing an otherwise normally occurring inhibitor. Goldwasser (65) showed that excised tissues including thymus, spleen, stomach, intestines, adrenals, pancreas, gonads, and 90 per cent of the liver did not eliminate the ability of animals to respond to bleeding or cobalt ion and thereby inducing erythropoiesis. Crafts (27) noted that neither subcutaneous injections of 0.25 unit of liver extract when given to hypophysectomized rats, or doubling the dose on the 30th day were effective in stimulating erythropoiesis. Gordon and co-workers (74) found that continuous administration to normal rats of boiled filtrates of plasma and peripheral red blood cells obtained from repeatedly bled rabbits resulted in slight but significant increases in red blood cell counts, hemoglobin, and hematocrit values. Prentice and Mirand (127) stated that liver damage, depending upon the amount of liver damage, would excite erythropoiesis. However, when the liver was acutely damaged with carbon tetrachloride and they were then placed in low oxygen atmosphere, significant amounts of such an erythropoietic factor appeared in the plasma (126). Gordon and co-workers (76) detected

slight activity in the liver, but other experiments appear to nullify these organs, presumably blood forming in nature.

Tissues. Relatively few investigators have attempted to locate the site of formation of the blood erythropoietic factor. Glant and Root (79) gave first proof of tissue extract stimulation of erythropoiesis comparable to that induced by hypoxia. Gordon (76) collected and used filtrates of livers, spleens (96), thymus, lungs, brains (except medulla and pituitary), gastrocnemius muscle, bone marrow, and packed blood cells. Among the different materials used, only a plasma extract stimulated erythropoiesis within the femoral bone marrow. Ramback, Alt, and Cooper (128), using Fe^{59} and P^{32} , revealed the erythropoietin reserve of the spleen of the rat under demand is an active participant in erythropoiesis and was equal to 16 per cent of the total marrow. The evidence for an endocrine function of the above for erythropoiesis stimulation is meager. In this respect the spleen is still Galen's "organ of mystery."

Milk and Urine. Grant (78) introduced milk as a source of the erythropoietic factor; that is, a substance was present in milk of anoxic rats and mice which produced an increased hemoglobin content of the nursing young. Other body fluids such as urine have been experimentally tested in both humans and animals. Winkert and associates (171) compared anemic human plasma, and found anemic human urine was more dilute and a less constant source of an erythropoietically active material. Hodgson and co-workers (87) discovered that urine from phenylhydrazine-

treated rats was a valuable source of erythropoietin for study and was not difficult to obtain.

Pillero, et al. (124), Hodgson and Toha' (87), and Winkert, et al. (171) proposed that urinary erythropoietin was potent, but less so than plasma erythropoietin. Winkert and associates (171) stated that the urinary erythropoietin did increase the hematocrit value and increased circulating red cell volume. Toha' and Hodgson (87) concluded that plasma and urine of animals with increased erythropoiesis contain a factor or factors (erythropoietins) which were probably the humoral mediators of the primary erythropoietic stimulus. Van Dyke, Garcia, and Lawrence (158) emphasized that the erythropoietin factor of urine of patients with aplastic anemia was equally as potent as the plasma erythropoietic factor. Thus, it may be postulated that the source of the erythropoietic material found in plasma and urine were identical but it was not yet proven that they arose from the same tissue.

Minerals and Vitamins. The essential metabolites such as iron, folic acid, vitamin B₁₂, cobalt, zinc, and copper are needed in the formation of red blood cells, but the erythropoietin is required to maintain the status of health of the red cell mass or the erythron in the body. Crafts (32) has shown that three minerals: iron, copper, and cobalt are essential for normal erythropoiesis. Cartwright (17) stated that a deficiency in copper produced an anemia characterized by a decreased red cell life span, as well as a decreased rate of production of

erythrocytes. It has been known for almost three decades that cobalt increases erythropoiesis in experimental animals (106). The precise mechanism by which cobalt exerts its action has not been fully clarified and is still problematic. Orten and associates (122) have shown quite conclusively that cobalt injections did not cause a hemoconcentration. Thus, the polycythemia was not due to changes in plasma volume. Berwald, Arsenan, and Dooley (6) claimed that cobalt would not induce a polycythemia in the absence of the spleen, but subsequent work by Orten (119) tended to disprove this role of the spleen. Korst and Bethell (99) emphasized that the action of cobalt did not appear to be altered by splenectomy.

There was an indication that cobalt interfered with cellular respiration to such an extent that an internal anoxia was produced. This anoxia, in turn, stimulates the bone marrow to produce more erythrocytes. Barron and Barron (4) postulated that cobalt interfered with respiration of the erythrocytes themselves, and therefore, an anoxic state developed. Warren, Schubmehl, and Wood (166); Orten and Bucciero (121); and Bucciero and Orten (14) have found that the respiration of the erythrocytes was normal and that the interference was at the level of tissues to be oxygenated. Wesley (170) substantiated this theory in which he found that methylene blue (known to enhance internal cellular respiration) would counteract the action of cobalt. Crafts (22) noted that it seemed likely that cobalt enhanced, in some manner, the utilization of iron reserves in the formation of hemoglobin.

Goldwasser and associates (68) indicated strongly that cobalt enhanced red-cell production by increasing the formation of erythropoietin. They suggested that cobalt plasma and anemic plasma contained erythropoietic factors with grossly similar properties, so when erythropoietin had been characterized more fully, it would be possible to determine whether cobalt plasma contained the factor identical with that found in anemic plasma (37). Goldwasser and co-workers (66) showed that the erythropoietic effect produced by cobaltous ion was probably caused by an increase in circulating plasma erythropoietin. The mechanism was obscure, but the authors suggested that production of erythropoietin was stimulated by a relative anoxia, and that cobalt may act by producing an anoxic state in the kidney (68) rather than in the bone marrow; this was also agreed to by Warren, et al. (166).

Goldwasser and co-workers (66) proposed that these findings are of interest from the clinical point of view. If the erythropoietic effect of cobalt induces an increase in plasma erythropoietin, then the clinical usefulness of the hormone is obvious. There are a number of disease conditions and experimental states in which the associated anemia is reported to be influenced beneficially by the administration of cobalt. Cobalt will favorably influence the anemia of hypophysectomy (27), protein deficiency (120), chronic renal disease (59), chronic inflammation (173), and cancer (139). Weisbecker (169) claimed that cobalt treatment was beneficial in anemias of infections,

anemias due to blood loss, iron deficiency anemias, and anemias due to tumors, but it was not particularly effective in aplastic anemia or macrocytic anemias.

The rapidity of the response to cobalt was important in the search for the site of production of erythropoietin. Goldwasser and associates (68) have shown that plasma from animals that have been injected with cobaltous chloride rapidly developed a high titer of erythropoietin. Brown and Meineke (13) found that injections of plasma obtained from "cobalt treated" animals increased the level of circulating reticulocytes from 36.4 to 54.4 per cent above values obtained from injecting normal plasma. Erythropoietic activity of plasma was increased in rats receiving prolonged cobalt therapy. The activity was not due to increased levels of cobalt in the plasma containing erythropoietin. Plasma from cobalt-treated animals had less erythropoietic activity than plasma obtained from animals that had been bled (13).

Amino Acids. Cartwright (16) divided the known factors concerned in erythropoiesis into vitamins, amino acids, and minerals. The minerals have already been reviewed. He reported the following vitamins play a role in this process in at least one animal species: riboflavin, nicotinic acid, pyridoxine, Lactobacillus casei group, extrinsic factor (vitamin B₁₂), ascorbic acid, pantothenic acid, and biotin. The writer felt that folic acid should be added to this group due to its role with iron incorporation into the red blood cell and vitamin B₁₂. The globin fraction of

the hemoglobin molecule is known to contain many of the so-called essential amino acids and many of those called non-essential. The amino acids: tryptophane, lysine, phenylalanine, isoleucine, and glycine have received the greatest attention. Korst and Bethell (99) stated that the action of cobalt was not significantly modified by administration of cysteine or histidine. Crafts (27) said that vitamin B₁₂ was ineffective in the stimulation of erythropoiesis.

Drugs Depressing and Stimulating Erythropoiesis. Erslev (79) theorized that chloromycetin depressed and developed an erythropoietic hypoplasia with a prompt hematologic recovery with well-defined reticulocytosis after discontinuance of the drug. Erslev (41) indicated that colchicine delays but did not abolish the reticulocyte response. Fried and co-workers (55) administered dinitrophenol to normal rats that resulted in increased rate of erythropoiesis about 70 per cent greater than controls, and that triiodothyronine gave similar results. Jacobson and co-workers (95) suggested that bleeding and the administration of phenylhydrazine, dinitrophenol, or triiodothyronine increased the production of erythropoietin. In these conditions, contrary to those in which a decreased erythropoietin production occurs, the demand for O₂ was increased whereas the supply remained normal (dinitrophenol and triiodothyronine), demand was unchanged, and the O₂ supply was reduced (bleeding or phenylhydrazine).

Eosinophilia. Gordon, et al. (74) indicated that eosinophilia was the sole peripheral leucocytic effect induced by the plasma, blood cell, and liver extracts obtained from bled rabbits. Militating against a non-specific or antigenic action here was the finding that no such increase in peripheral eosinophil numbers occurred following treatment with similarly prepared extracts of normal plasma and blood cells. The precise significance of this eosinophilia and its relation to the erythrocytic changes remain to be investigated.

Humoral Theory

The renewed interest in the humoral control of erythropoiesis in the past few years has led to great advances in our knowledge of the mechanisms that govern red cell production. Carnot and Deflandre, in 1906 (15), demonstrated an erythropoietic factor in serum of rabbits rendered anemic by bleeding. Such plasma injected into other rabbits resulted in a hyperplastic bone marrow and increased reticulocyte and erythrocyte counts. The investigation prior to 1952 was reviewed by Grant and Root (79), at which time the evidence pointed toward the humoral control of erythropoiesis. These erythropoietic factors were called Erythropoietin by Bonsdorff and Jalavisto (10).

Several investigators (9, 37, 38, 42, 43, 63, 74, 75, 76, 80, 84, 98, 101, 107, 123, 144, 147) have obtained some erythropoietic stimulating activity from serum or plasma by exsanguination to render the rats and rabbits anemic. Linman and Bethell (104,

106); Gordon, et al. (69, 70, 73, 75, 76); Borsook, et al. (11); and Lowy, et al. (109) rendered their animals anemic by phenylhydrazine injections. Gordon and Dubin in 1934 (72) disagreed with this theory, using the reticulocyte index which was not available to the earlier workers, and reported no change in either the number of erythrocytes or in the reticulocyte percentages. The negative results with the use of "anemic" serum or plasma were explained by Erslev (42) in that larger quantities of the "anemic" plasma were necessary to produce the erythropoietic activity. An important advancement by Borsook, et al. (11) was made when they demonstrated an erythropoietic-stimulating factor in the filtrate obtained from heat-denatured anemic rabbit plasma after deproteinization by boiling for 10 minutes at pH 5.5. Subsequently, many investigators (69, 74, 75, 76, 96, 97, 103, 116, 124, 129) reported similar observations. Erslev (45) and Stohlman and Brecher (143, 144) found plasma so treated to be ineffective. Jacobson (96) indicated that boiled plasma from animals with phenylhydrazine treatment did not produce a uniform response, and the erythropoietic activity could be demonstrated in boiled plasma only if the phenylhydrazine intoxication had been severe enough to induce significant liver damage.

Fasted or starved rats were used as test animals by Hodgson, et al. (89) and Fried and associates (55). These animals, subjected to starvation, have a decreased basal metabolic rate, and a marked decrease in tissue demand of oxygen exists without appreciable change in the number of circulating erythrocytes.

Thus, a relative plethora of red cells existed in these animals. Jacobson, et al. (96) reported that plasma from rats starved for four days had no erythropoietic activity, but erythropoietic activity was present in the plasma of pregnant mice during the last trimester of pregnancy. The use of hypophysectomized rats produced a greater sensitivity to anemic plasma than a normal rat since after hypophysectomy, the overall metabolic requirement of the animal dropped rapidly to a level that was a fraction of that in the normal animal (55). Jacobson, et al. (92) assumed, for the sake of simplicity, that the factor(s) in normal plasma and in the plasma of normal or hypophysectomized animals subjected to bleeding that stimulated erythropoiesis in hypophysectomized and in polycythemic assay preparations were one and the same. Hypophysectomy and polycythemia are analogous in that both result in a decreased rate of erythropoiesis and increased responsiveness to anemic plasma (55). Different physiological conditions as stressors affected the erythropoietic stimulation activity in plasma (21), as the humoral erythropoietic factor was increased markedly during pregnancy.

Some idiosyncracies to pregnancy were observed in the following experiments. Erythropoietin in pregnant transfusion-induced polycythemic mice was suppressed throughout pregnancy, and erythropoietin production was concomitantly suppressed (91). This agreed with Fired, et al. (55) who observed that the rate of erythropoiesis was proportional to the amount of erythropoietin produced. Erythropoietin in the fetuses of these mothers was

induced in ten days and maintained. At birth, the hematocrit was higher than normal, showing the fetus to be independent and acquiring stimulation through the increase of iron from the breakdown of transfused cells. Seip (138) injected normal human infants with adult plasma, newborn normal plasma, and plasma of infants with erythroblastosis. The latter two injectants caused an increase in the reticulocyte count.

Toha' and co-workers (153) stated that the injections of plasma from rabbits anemiated by bleeding produced a significant increase of the reticulocytes, red cells, and hemoglobin in the normal animal. Underbjerg and associates (155) reported this phenomenon with plasma filtrate of anemiated bovine plasma, which produced a reticulocytosis at an average of 283 per cent, as compared to 69 per cent in normal bovine plasma. Lowy and co-workers (108) stated that a true polycythemia can be produced in normal adult rats at ordinary altitudes by injection of the filtrate of boiled plasma of rabbits made severely anemic with phenylhydrazine. All criteria of polycythemia were observed: increases in red cell count, hematocrit, and hemoglobin concentration, as well as a reticulocytosis and evidence in the bone marrow of stimulation of erythropoiesis.

Iron depletion (anemia or hemorrhage) was characterized by a more rapid and more complete utilization of radioactive iron. If, on the other hand, storage iron was not greatly altered, the percentage utilization was determined by the function of the erythropoietic tissue (49). Hahn and co-workers (85) stated that

there was no evidence of any exchange between the hemoglobin iron and radioactive iron. Peacock, et al. (123) expressed belief that there was no exchange of radioactive iron in the red blood cells.

Walsh and co-workers (164) reported on a radioactive iron technique that the exchange of iron between the mature erythrocyte and surrounding plasma was negligible. Wasserman and associates (167) suggested that with normal body iron stores, the half-time of plasma radioiron disappearance was a sensitive measure of the integrity of the erythropoietic tissue of the body. Hemoglobin synthesis was more sensitive as an indicator of erythropoiesis than oxygen consumption, as was illustrated by the considerably greater stimulation effect of normal serum (150). Finch and co-workers (49) confirmed this by stating that free exchange of iron did not occur between erythrocytes and plasma or the tissues.

Nature of the Erythropoietic Factor(s)

There have been many varied observations on the possible nature of the humoral erythropoietic factor(s). Erslev and Laviates (48) assumed that the erythropoietic factor was attached to, or behaved like, a serum albumin, alpha globulin, or beta globulin. Brecher (12) believed that the erythropoietic factor was a mucoprotein; that is, a substance with an electrophoretic mobility between alpha-1 and alpha-2 globulin. Ramback (128) demonstrated that the erythropoietic activity was associated with a protein having the electrophoretic characteristics of an

alpha-2 globulin because the globulin was not precipitated by perchloric acid or boiling; it was heat stable, stained for carbohydrate and not for fat, contained nitrogen, and appeared to be of the class of mucoproteins.

Brecher (12) stated that Linman and Bethell believed the heat stable factor stimulated cell division without a change in the hematocrit and hemoglobin. Linman and Bethell further suggested that the heat stable factor was batyl alcohol or closely related to it. Watson of Minnesota (168) indicated that batyl alcohol (132) was likely to be a hemolytic agent which may explain Linman and Bethell's result of reticulocytosis and possibly sperocytosis, without changes in hematocrit and hemoglobin; in short, a compensated hemolytic anemia of a mild degree. It was stated (12) that the daily doses of 12.5 and 25 mg of batyl alcohol used by Linman and Bethell was far more than could possibly be present in any plasma.

Ramback (128) reported that the erythropoietic factor was not dialyzable, was precipitated by 75 per cent saturation with ammonium sulfate, and was not extractable from the lyophilized state by repeated washings with ether. It does not diminish in storage at 5° C. in the liquid state for one year, or in the lyophilized state at room temperature for six months, and was not removed by passing through Seitz or Berkefeld filters. Robbins (134) believed that it may be a mucoprotein that acts for an acidic glycoprotein in the transport of thyroxine. Toha' and associates (153, 154) suggested that it may be a fatty acid ester

and not an amino acid, purine, or sugar. Slaunwhite and co-workers (141) maintained, with evidence, that the erythropoietic factor was a low molecular weight protein, polysaccharide, or polypeptide, perhaps in range of insulin or ribonuclease. In the presence of the nonprecipitable proteins (by heat) of plasma, it was fairly stable to mild oxidation and reduction. Ramback and associates (131) announced that no carbohydrate could be found on paper chromatograms and this mucoprotein loses its erythropoietic activity upon hydrolytic removal of neuraminic acid. Ramback (130, 131) has shown that the erythropoietic factor prepared to be of a low molecular weight, acidic glycoprotein, using DEAE-cellulose ion-exchange columns, Gordon (71) also stated that it was a mucoprotein or moiety of elevated mucoprotein.

There are several possible explanations for the discrepancies on the nature of the material and its effects. As with other hormones, we were dealing with a target organ(s), different methods of demonstrating erythropoietic stimulation, source and species differences, various techniques utilized in preparing the extracts, and the possibility of the existence of more than one factor. The concept that there was more than one humoral factor which induces erythropoiesis was not a new one. Tei (149) reported that the plasma of phenylhydrazine-treated and bled rabbits produced an erythropoietic factor that resisted boiling; was acetone, ether, and alcohol soluble; and postulated that it probably was a lipid. He also described another factor in the

plasma of animals subjected to reduced oxygen tension or fed garlic that was thermolabile, ether insoluble, and probably a globulin.

Linman and Bethell (106) indicated in their studies, the existence of at least two plasma erythropoietic factors. One was heat-stable, ether-soluble, and most likely a lipid. This factor appeared to increase the rate of cellular division of already existent marrow erythrocytic precursors with resultant erythrocytosis due to the production of microcytes with shortened survival times. The heat-stable, ether-soluble factor did not accelerate iron⁵⁹ incorporation in hemoglobin and did not increase the total circulating hemoglobin or red cell mass. The other factor, which was relatively thermolabile, ether insoluble, and probably protein in nature, augmented iron incorporation and hemoglobin synthesis. Gley (64) also described two factors in "anemic" serum. He believed one to be a trioxomolalcoholic sterol and the other to have properties closer to the protein group. Van Dyke, et al. (16) described two fractions of erythropoietin. The first, soluble in acetone, was called hematopoietine and the second, acetone insoluble, which activated the first fraction, was called hematostimuline. Stohlman and Brecher (145) did not warrant the assumption of a separate heat stable component, but did not entirely exclude this possibility. Mirand and Prentice (116) appeared to be justified for the present, to consider that there was a single humoral factor of which a major portion was destroyed by boiling.

Mode of Actions of the Erythropoietic Factor(s)

Whether the erythropoietic factor(s) affected the erythropoietic system by increasing cell division, the rate of maturation, delivery from hematopoietic centers, or by concomitantly accelerating all three processes has not been completely answered. Physiologic reports indicated that the plasma erythropoietic-stimulating factor(s) produced erythroblastic multiplication rather than an increased maturation (39). Linman and Bethell (106) stated that the heat-stable, ether-soluble, and likely a protein factor appeared to increase the rate of cellular division of already existent marrow erythrocytic precursors with resultant erythrocytosis due to the production of microcytes with shortened survival times. It did not accelerate iron⁵⁹ incorporation in hemoglobin and did not increase the total circulating hemoglobin or red cell mass. The other factor which is relatively thermostable, ether-soluble, and probably protein in nature, augmented iron incorporation and hemoglobin synthesis. These factors, when given to normal rats, produced erythrocytosis due to microcytes, reticulocytosis, and myeloiderythrocytic hyperplasia with no associated increases in hemoglobin or hematocrit values. Linman and Bethell (105) found in the recipient rat, that this erythropoietic stimulation was manifested by erythrocytosis, reticulocytosis, and increased marrow erythropoietic activity, and suggested that specie differences and the duration of erythropoietic stimulation may determine an increase in the red cell mass.

Hypoxia was believed to be the stimuli to the formation of the humoral factor(s) in patients with secondary polycythemia. Stohlman and Brecher (144, 147) stated that hematologic studies of both human beings and experimental animals suggested the possibility that erythropoietin could be produced and controlled by a body mechanism functioning independently of the hypoxic process. The second mechanism involved an inhibitor of red cell formation produced in senescent red cells and released on death of such cells, and postulated that the amount of inhibitor so released could be reduced either by hemorrhage or by hemolysis before red cells reach senescence. Ramback (128) demonstrated a rise in reticulocytes, hemoglobin, and hematocrit following the injection of "anemic" protein filtrate, and this induces an increased rate of delivery of red cells from the bone marrow. Erslev's (47) observations indicated that the anoxic stimulus operates in the bone marrow by accelerating the differentiation of stem cells into pronormoblasts and that thereafter the maturation and multiplication of differentiated nucleated red cells proceeded at fixed rates independent of the anoxic stimulus. In summary, the mode of action of erythropoietin was to control the rate of cell division, cell maturation, and delivery of the cell from the erythropoietic centers.

Assay and Methods

In vivo Fe^{59} uptake was first demonstrated in rat bone marrow by film strip cytoautoradiography of marrow smears after injection

of radioactive iron (2, 3). Austoni (3), employing film strip audioradiography after intraperitoneal injection of labeled ferric chlorides, elicited that pro-erythroblasts and basophilic erythroblasts took up a detectable amount of radio-iron after three to six hours, but that the maximum uptake occurred in the polychromatic and orthochromatic stages. It has been determined by histologic studies in mice (8), that the normoblast was the most radiosensitive marrow cell.

Radioactive iron, given enterally or parenterally was shown by Hahn, et al. (85) to be incorporated into the red blood cell as an integral part of the hemoglobin molecule. It was stated that there was no exchange of iron between hemoglobin iron and the radioactive iron in the solution, or between the mature erythrocyte and surrounding plasma (49, 50, 61, 62, 85, 123). Walsh and colleagues (164) described the physiological process of the assimilation of iron to acceptors in the red cell stroma capable of removing iron from the serum and secondarily, the synthesis of heme.

It was reported by Peacock and co-workers (123) that it was generally believed that hemoglobin was incorporated inside the developing erythrocyte. If some of the iron atoms involved in this synthesis were radioactive, a proportionate number of them would become an integral part of the new hemoglobin molecule with the newly developed red blood cell. Following the release of the cell into the circulation, its presence in the blood stream could be detected as long as it remained morphologically

intact. When the tagged red cell was destroyed, the hemoglobin-derived iron was very rapidly removed from the plasma, to be reused, to some extent at least, in the synthesis of new hemoglobin. Generally, each red blood cell contained approximately 0.1 per cent iron, or about one thousand million atoms of iron.

Recent investigators have used these techniques as assay methods, or the iron turnover in plasma and red cells was determined by using tracer amounts of Fe^{59} in 1952 by Elmlinger, et al. (40). The determination of iron absorption, using two isotopes, Fe^{55} and Fe^{59} , was utilized by Saylor and Finch (137) and Gibson (61). Plzak and associates (125), using Fe^{59} , demonstrated the stimulation of erythropoiesis in plasma from anemic rats. A test for erythropoiesis, employing deproteinized plasma extract along with Fe^{59} , enabled cross-species examination of erythropoietic factor in plasma (116). Mirand and Prentice (118) gave proof of the validity of radioactive iron in determining red cell production in mice. It was shown that plasma iron turnover was a good index of hemoglobin synthesis. Hodgson and co-workers (88) stated that the use of a fraction of Fe^{59} appearing in red cells at 24 and 48 hours, was not a good index of the state of erythropoiesis. Iron⁵⁹ was used by Stohlman and Brecher (142) in their study of humoral regulation of erythropoiesis and the relative heat stability of erythropoietin, and by other workers (145). Gurney, et al. (83) employed Fe^{59} as a basis for their studies on erythropoiesis, employing human plasma, and on the theoretical and clinical significance of the dynamic equilibrium of erythropoiesis. Goldwasser and associates (65,

66, 67, 68) utilized Fe^{59} in their studies on erythropoiesis with the aid of cobalt. Fried and co-workers (54) emphasized that Fe^{59} uptake in newly-formed red cells probably was the most reliable single method for studying the effect of anemic plasma on erythropoiesis.

An increase in the reticulocyte count following injection of anemic plasma in recipient animals was used as a criterion by many investigators prior to the employment of Fe^{59} . Some of the investigators using this method were: Crafts (33); Meineke and Crafts (111, 112, 113); Toha', et al. (153); Erslev (46); Jacobson and associates (91, 94); Grant (78); and Gordon, et al. (76). Giblett and co-workers (6) used both reticulocytes and Fe^{59} as did Linman and Bethell (104). Other research investigators: Crafts (27, 28, 29); Crafts and Meineke (35); and Silbergleit (140) applied only the complete hemogram as a basis for comparison.

Jacobson and associates (96), in their investigations, elicited the following new aspects on erythropoiesis: a) That the embryo of the transfusion-induced polycythemic mouse had the capacity to initiate and maintain erythropoiesis, although erythropoietin(s) did not appear to be available from the maternal circulation; b) That little or no erythropoietin passed from the fetus to the mother because reticulocytes had not appeared in the maternal circulation, but that the dilution factor in the maternal plasma was so great that it precluded a recognizable effect; c) That young laboratory mice gave birth to anemic infants

and that this could be remedied by the administration of iron or red cell transfusion to the mothers; and d) That it was also possible for the fetuses to initiate and maintain erythropoietin independently from the maternal circulation. Contopoulos and co-workers (21) reported the humoral erythropoietic factor was markedly increased during pregnancy, and that injections of plasma from non-pregnant rats resulted in an increase in plasma and blood values of hypopsectomized and newborn recipients.

Effect of Irradiation on Erythropoiesis

The hemopoietic tissue of rabbits was not affected by irradiation in the same manner as it was with nitrogen mustard (48), since the former did not impair the production of the erythropoietic-stimulating factor. Protein-free plasma extracts from anemic rabbits, immediately following total body x-irradiation, are capable of stimulating erythropoiesis in the normal rat as demonstrated by erythrocytosis, reticulocytosis, and increased marrow erythropoietic activity (104). Stohlman and associates (146) established the fact that following whole body exposure to sublethal doses of X-rays, erythropoiesis was depressed both in dogs and rats, but could be markedly increased by bleeding the animals shortly before or after irradiation; however, blood loss 24 hours after irradiation had no appreciable effect. Linman and Bethell (104) reported that plasma extracts from rabbits made anemic by total body x-irradiation alone contained an erythropoietic stimulating factor. These data indicated that the

stimulating factor was not produced by hemopoietic or other radioactive sensitive tissue, and its formation was not dependent upon a regenerative marrow. Valentine (156) stated that suppression of erythropoiesis in cats given sublethal doses of X-ray could not be demonstrated.

Stohlman and Brecher (142) demonstrated an increased sensitivity in erythropoiesis of sublethally-irradiated animals which was of considerable importance. Whereas multiple injections of anemic plasma are required to evoke an increase in red cell production in normal rats, only a single injection was necessary in the sublethally-irradiated animal. The increased sensitivity of these animals to erythropoietin may be the consequence of a diminution in a normally-occurring inhibitor. Stohlman and Brecher (143) proposed that the sublethally-irradiated rat may serve as a useful animal for the further investigation of the regulation of erythropoiesis. The method employed required a single irradiation treatment, allowed a satisfactory estimate of red cell production by the Fe^{59} incorporation technic, and required only five days for an experiment. This was in agreement with the findings of Henessey and Huff (86).

Condition of Anemic Patients

Recently, investigators have turned to the significance of the presence of erythropoietin in different anemic states (30), especially in the human being. Piliero (124) and co-workers, employing boiled filtrates from patients with Cooley's anemia

and sickle cell anemia, stimulated production of erythropoietin in normal rats. The extract of urine from one of Cooley's anemia patients showed potency of erythropoietin, but the urine of patients with chronic hypoplastic anemia was inactive. Van Dyke and associates (158) stated that patients with aplastic anemia had an exceptionally high level of erythropoietic activity in plasma as well as in the urine. Patients with aplastic anemia demonstrated erythropoietin-stimulating properties in their plasma although two plasma specimens obtained following transfusion were negative (81).

Gurney (81, 82) reported that plasma of four out of six leukemic patients showed presence of erythropoietin. Positive results were obtained with extracts of plasma from patients with pernicious anemia, acute gastrointestinal hemorrhage, and Hodgkin's Disease, but anemic patients with carcinoma, chronic nephritis, plasmocytic myeloma, or sickle-cell anemia failed to produce evidence of the erythropoietin-stimulating factor. Erythropoietic activity was demonstrated in the plasma from patients with pernicious anemia, blood loss anemia, aleukemic leukemia, Hodgkin's Disease, acute hemolytic anemia, or hypoplastic anemia, but was not found in patients with uremia, advanced neoplasms, or malnutrition (84).

Finch and colleagues (49) found a correlation between a variety of hematologic disorders such as iron deficiency; blood loss anemia; hemochromatosis; refractory, aplastic, and myelophthisic anemias; uremia; viral infections; bacterial,

protozoal, and hemolytic anemia; pernicious anemia; malaria; malignancy; endocrine disease; Addison's; acute infections hepatitis; Laennec's cirrhosis; obstructive jaundice; polycythemia vera; chronic congestive heart failure; and secondary polycythemia. The results of these studies were consistent with the hypothesis that the rate of erythropoiesis was mediated by the humoral mechanism.

MATERIALS AND METHODS

Source Material

Twelve yearling Hereford heifers, averaging 823 pounds, were utilized as source material for erythropoietin. The animals were maintained on a balanced ration consisting of alfalfa, corn, linseed oil meal, meat scraps, calcium carbonate, dicalcium phosphate, and iodized salt. The roughage was fed ad libitum and the concentrates were supplied so that the animals would gain an average of two pounds per day. These animals were anemiated by exsanguination weekly for seven weeks according to their hemogram. The cattle were bled at the rate of 15 per cent of their total blood volume; the latter being considered as 8 per cent of their body weight.

Preparation of Partial Protein-free Filtrate

The site of incision was injected with a 2 per cent procaine solution for the local anesthesia prior to bleeding. The skin was incised over the jugular vein and a trochar was inserted into

the vessel. A sterile plastic tube that had been rinsed with an anti-coagulant, A. C. D. solution,¹ was placed on the trochar connecting it with the collecting bottle containing A. C. D. solution. Aseptic precaution was adhered to throughout the bleeding period. A blood sample was obtained to procure a complete hemogram from each animal at each weekly bleeding. The origin of the source material with the number of bleedings and the average hemogram is presented in Table 1.

Table 1. Origin of source material of partial protein-free filtrate from anemiated cattle with average hemogram.¹

Source material:	Hematocrit ²	Hemoglobin ³	Red blood cell ⁴
1	46.4	11.51	8,348,000
2	36.4	9.85	6,393,000
3	32.0	8.58	5,393,000
4	28.7	8.15	4,882,000
5	27.2	6.98	4,694,000
6	23.2	6.20	4,363,000
7	22.2	5.99	4,148,000

¹ Pooled samples of 12 Hereford heifers.

² Ml. of red blood cells per 100 ml. of blood.

³ Gm. per 100 ml. of blood.

⁴ Millions of red blood cells per cc³.

The blood was stored in a walk-in refrigerator in case it was not processed the same day. Processing consisted of placing the

¹ An anticoagulant consisting of acid-citrate-dextrose.

blood in an International Harvester Model 3-S cream separator to separate the pooled plasma from the formed elements of the blood. The pooled plasma was then treated according to the technique described by Gordon and associates (76); that is, the pooled plasma was acidified with 1N hydrochloric acid to a pH of 5.5. Following the acidification, the plasma was heated at 99.9°C . for 10 minutes to precipitate the proteins such as albumins, globulins, and fibrogen to obtain a partial protein-free filtrate (PPFF). The clear PPFF was stored aseptically in glass containers at 5°C . until it was used for experimental research.

Preparation of Assay Animals

Young virgin female rats of the Sprague-Dawley strain, ranging in weight from 150 to 185 gm. were used throughout the six experiments. They were maintained on a ration of Purina Laboratory Chow and fed ad libitum. The normal standardized rats were randomized and grouped according to their pre-injection hematocrit values, with 10 animals assigned to each group. The animals with low hematocrits were not utilized in these experiments as they tended to exhibit higher iron uptakes (141).

The experimental design with the number of the experiments, the number of bleedings, and the age of the source material, PPFF, at the time of assay for potency of erythropoietin is shown in Table 2. Each experiment was carried out over a period of four days. The experiments were completed in approximately three weeks.

Table 2. Experimental design with the number of experiments, the number of bleedings, and the age of source material, PPFF, at time of assay for potency of erythropoietin.

Experiments	: : Number : of rats	: : Bleeding ¹	: : Age of : Source material : (days)
1	10	2	309
	10	1	315
2	10	3	305
	10	1	318
3	10	4	301
	10	1	321
4	10	5	297
	10	1	324
5	10	6	293
	10	1	327
6	10	7	289
	10	1	330

¹ Bleeding-source material (PPFF) tested for erythropoietic potency.

The PPFF was administered according to the technique employed by Fried and colleagues (54). Two ml. of the PPFF was injected intraperitoneally daily for three consecutive days. Two hours following the last injection of the PPFF, 1 ml. of $\text{Fe}^{59}\text{Cl}_3$, diluted in normal physiological saline containing approximately three microcuries of radioactive Fe^{59} was administered intracardially into the recipient animals under ether anesthesia. The radioactivity in an aliquot of the original $\text{Fe}^{59}\text{Cl}_3$ solution given each animal was measured to allow for the half-life that was lost during the six experiments.

Nineteen hours following the administration of the $\text{Fe}^{59}\text{Cl}_3$ physiological saline solution, a 1 ml. sample of blood was withdrawn from each rat (both the 10 treated and the controls) by cardiac puncture under ether anesthesia. The modification of Fried's technique was employed as a better criterion of $\text{Fe}^{59}\text{Cl}_3$ uptake in the erythrocyte (54). The radioactivity of the uptake of $\text{Fe}^{59}\text{Cl}_3$, incorporated into the newly-formed erythrocytes in these samples, was measured in a G. M. Dipping Tube Counter with a Berkeley Decimal Scaler model 210-S. The percentage of radioactive uptake of $\text{Fe}^{59}\text{Cl}_3$ in the 10 treated rats with PFFF-2 through 7 was compared to the 10 control rats injected with PFFF-1 in each of the six experiments.

RESULTS

Results of Experiments for Potency of Erythropoietin

The results of the experiments on the assay of the potency of erythropoietin in cattle blood and the data on each individual rat, as outlined in the experimental design in Table 2, are delineated in Tables 3 to 14, inclusive. The pertinent data presented are self-explanatory. All data were then subjected to a statistical analysis. The results of the analysis of the six experiments, Tables 3 to 14 inclusive, are presented in Table 15.

Table 3. Experiment 1, Group I: Effect of PPFF-2 upon erythropoiesis measured by $\text{Fe}^{59}\text{Cl}_3$ uptake in the erythron of rats.

Animal number	Weight :in grams:	Hematocrit pre- : Post- :	Amount : injected : : (cc.) :	Amount : in γ c : : sample :	C/M of : standard : : sample :	C/M of : blood : : sample :	Back- : ground : : C/M :		
2	168	49.0	40.0	6	2.92	236	200	76	124
7	166	49.0	29.0	6	2.92	236	173	76	97
8	174	49.0	42.5	6	2.92	332	246	74	172
10	166	46.0	42.0	6	2.92	332	200	74	126
19	166	44.0	39.5	6	2.92	236	177	76	101
26	163	43.0	29.0	6	2.92	236	233	76	157
28	182	44.5	40.5	6	2.92	236	173	76	97
32	157	40.0	39.5	6	2.92	236	175	76	99
43	167	41.5	39.0	6	2.92	236	209	76	133
47	170	46.0	40.0	6	2.92	236	232	76	156
Average	167.9	45.2	38.1				201.8	75.6	126.2

Table 4. Experiment 1, Group II: Effect of PPFF-1 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : : PPFF : Amount : : injected : Fe $^{59}\text{Cl}_3$:standard: blood : Back- : (cc.) : in % : sample : sample : ground : C/M	C/M of : C/M of : C/M	Net C/M
9	168	44.0 44.0	6 2.92	236 205	76 129
14	162	44.0 32.5	6 2.92	236 209	76 133
16	160	45.0 42.5	6 2.92	236 215	76 139
17	170	47.5 27.0	6 2.92	236 179	76 103
22	172	48.5 41.0	6 2.92	236 184	76 108
23	178	47.0 42.5	6 2.92	236 175	76 99
35	168	43.0 40.0	6 2.92	236 173	76 97
37	164	43.0 45.0	6 2.92	236 203	76 127
42	174	42.0 41.5	6 2.92	332 167	74 93
46	160	47.0 45.5	6 2.92	236 208	76 132
Average	167.6	45.1 40.15		198.8	75.8 116.0

Table 5. Experiment 2, Group III: Effect of PPF-3 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : PPFF : injected : (cc.)	Amount : Fe ⁵⁹ Cl ₃ : in % : sample	C/M of : standard : sample	C/M of : blood : sample	Back- : Ground : C/M	Net C/M
6	164	45.0 43.5	6	2.90	226	256	81	175
13	180	45.5 47.0	6	2.90	201	248	81	167
25	176	45.0 42.5	6	2.90	201	236	81	155
30	168	41.5 43.5	6	2.90	201	245	81	164
31	180	49.0 45.5	6	2.90	201	220	81	139
33	160	40.0 48.0	6	2.90	201	227	81	146
34	172	43.0 47.0	6	2.90	201	234	81	153
36	156	49.0 47.5	6	2.90	226	239	83	156
38	164	44.0 46.5	6	2.90	201	197	81	116
41	168	46.0 40.0	6	2.90	201	274	81	193
Average	168.8	44.8 46.1				237.4	81.2	156.4

Table 6. Experiment 2, Group IV: Effect of PPF-1 upon erythropoiesis measured by $\text{Fe}^{59}\text{Cl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit : Pre- : Post-	Amount : PPFP : injected : (cc.) : in %	Amount : C/M of : $\text{Fe}^{59}\text{Cl}_3$: standard :	C/M of : sample :	Back- : ground :	Net : C/M
3	170	46.0 44.0	6 2.90	201	196	81	115
4	174	46.0 45.5	6 2.90	201	159	81	78
5	160	47.0 50.0	6 2.90	201	263	102	161
12	170	42.0 46.0	6 2.90	226	258	83	175
20	172	48.5 42.0	6 2.90	201	215	81	134
27	158	39.0 44.5	6 2.90	201	258	102	156
29	162	46.5 46.0	6 2.90	201	222	102	120
39	167	40.0 44.0	6 2.90	226	226	83	143
45	156	48.0 46.0	6 2.90	201	271	102	169
48	160	46.0 45.0	6 2.90	201	206	102	104
Average	164.9	44.9 45.3			227.4	91.9	135.5

Table 7. Experiment 3, Group V: Effect of pppf-4 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : : pppf : Amount : : injected: $^{59}\text{FeCl}_3$: standard: blood : : (cc.) : in μc : sample : sample :	C/M of : : C/M of : : C/M of :	Back- : : Ground : : C/M :
51	162	44.5 48.0	6 2.88 195	234	88 146
60	166	43.5 41.0	6 2.88 195	280	88 192
72	162	45.0 45.5	6 2.88 195	251	88 163
75	186	45.0 46.0	6 2.88 194	309	86 227
76	156	41.5 46.0	6 2.88 194	309	86 223
77	176	46.0 47.0	6 2.88 194	203	86 217
81	162	45.0 48.0	6 2.88 195	331	88 243
87	178	47.0 49.0	6 2.88 194	316	86 230
89	178	45.0 48.0	6 2.88 195	266	88 178
91	166	46.5 45.5	6 2.88 195	248	88 160
Average	169.2	44.9 46.4		285.3	87.4 197.9

Table 8. Experiment 3, Group VI: Effect of pPFF-1 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : :in grams	Hematoorit Pre- : Post- : :	Amount : : pPFF : : injected : : (cc.) :	Amount : : C/M of : : $\text{Fe}^{59}\text{Cl}_3$ standard : : in % : sample :	C/M of : : blood : : sample :	Back- : : ground : : C/M	Net C/M		
50	168	51.0	49.0	6	2.88	164	308	88	220
52	180	49.5	47.5	6	2.88	164	265	88	177
53	164	43.0	42.5	6	2.88	164	273	86	187
57	178	44.0	43.0	6	2.88	194	261	86	175
63	174	45.5	44.5	6	2.88	194	266	86	180
66	174	46.0	49.0	6	2.88	194	263	86	177
71	166	37.0	48.0	6	2.88	164	200	88	112
74	170	43.5	48.0	6	2.88	164	250	86	164
82	176	45.0	49.0	6	2.88	164	225	86	139
83	166	46.5	43.0	6	2.88	164	258	88	170
Average	171.6	45.1	46.4				256.9	86.8	170.1

Table 9. Experiment 4, Group VII: Effect of PPFF-5 upon erythropoiesis measured by $\text{Fe}^{59}\text{Cl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : : PPFF : : injected : (cc.) :	Amount : : $\text{Fe}^{59}\text{Cl}_3$: : in γo : : sample :	C/M of : : standard : : blood :	C/M of : : sample : : ground :	Back- : : ground : : C/M
55	168	49.0 48.5	6	2.96	146	327	84 243
61	168	45.0 48.0	6	2.96	148	276	86 190
62	174	41.0 47.5	6	2.96	146	269	86 183
67	182	41.0 47.5	6	2.96	148	261	88 173
70	188	47.5 49.0	6	2.96	148	255	72 183
78	170	45.0 48.0	6	2.96	146	297	88 209
80	172	43.5 40.0	6	2.96	146	488	116 372
84	170	41.5 47.5	6	2.96	148	259	88 171
86	174	43.0 41.0	6	2.96	146	454	88 366
90	170	45.0 49.5	6	2.96	148	380	102 278
Average	175.6	44.2 46.7				326.6	89.8 256.8

Table 10. Experiment 4, Group VIII: Effect of PPF-1 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams :	Hematocrit Pre- : Post- :	Amount : PPFF : injected : (cc.) :	Amount : Fe ⁵⁹ Cl ₃ : in % :	C/M of standard : blood : sample :	Back- : ground : C/M :	Net C/M :	
54	162	46.5 45.0	6	2.96	146	312	96	216
59	166	43.5 43.0	6	2.96	148	284	96	188
64	184	45.0 45.0	6	2.96	148	261	90	171
65	172	48.5 47.5	6	2.96	146	325	100	225
68	182	45.0 46.0	6	2.96	146	219	84	135
69	170	43.5 46.0	6	2.96	148	260	88	172
73	164	43.0 45.0	6	2.96	148	223	72	151
79	172	44.0 46.5	6	2.96	148	253	60	193
85	158	42.5 45.0	6	2.96	146	290	86	204
92	172	42.0 43.0	6	2.96	146	241	90	151
Average	170.2	44.4 45.2				266.8	86.2	180.6

Table 11. Experiment 5, Group IX: Effect of PFFF-6 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : injected : (cc.)	Amount : PFFF : in %	C/M of : standard : sample	C/M of : blood : sample	Back- : ground : C/M
100	166	41.0 45.0	6	2.92	143	301	104 197
108	170	46.0 45.0	6	2.92	141	326	100 226
109	164	45.5 48.0	6	2.92	141	342	112 230
113	174	40.0 45.0	6	2.92	140	354	116 238
116	164	40.0 49.0	6	2.92	141	353	86 267
118	192	45.0 48.0	6	2.92	143	292	86 206
129	176	47.5 46.0	6	2.92	143	303	81 222
132	164	43.0 45.5	6	2.92	140	327	116 211
133	180	50.0 47.0	6	2.92	141	330	72 258
140	156	42.0 44.0	6	2.92	141	431	120 311
Average	170.6	44.0 46.4				335.9	99.3 236.6

Table 12. Experiment 5, Group X: Effect of PPFF-1 upon erythropoiesis measured by $\text{Fe}^{59}\text{Cl}_3$ uptake in the erythron of rats.

Animal number	Weight : in grams	Hematocrit Pre- : Post-	Amount : : PPFF : injected : : (cc.) : in $\text{Fe}^{59}\text{Cl}_3$:	Amount : : C/M of : standard : : in $\text{Fe}^{59}\text{Cl}_3$: sample :	C/M of : : blood : sample :	Back- : : ground : C/M
94	170	45.5 49.0	6	2.92	140 292	108 184
103	160	47.5 48.0	6	2.92	142 263	100 163
107	178	41.0 48.0	6	2.98	144 236	72 174
110	176	43.0 46.0	6	2.92	141 231	100 131
115	172	47.5 43.5	6	2.92	142 282	100 182
122	166	45.5 44.0	6	2.92	144 232	100 132
123	170	43.0 46.0	6	2.92	142 226	102 124
127	168	43.0 42.5	6	2.92	140 235	96 139
131	166	44.0 43.0	6	2.92	142 258	96 162
138	180	44.0 46.0	6	2.92	141 246	80 166
Average	170.6	44.4 45.6			251.1 95.4	155.7

Table 14. Experiment 6, Group XII: Effect of PPFF-1 upon erythropoiesis measured by $^{59}\text{FeCl}_3$ uptake in the erythron of rats.

Animal number	Weight : :in grams:	Hematocrit : : Pre- : Post- :	Amount : : injected : (cc.) :	Amount : : C/M of : $^{59}\text{FeCl}_3$:standard: blood : sample :	C/M of : : Back- : ground : C/M :
95	171	47.0 41.5	6	2.98 144	263 104 159
96	160	46.0 43.5	6	2.98 144	238 65 173
98	162	42.0 26.0	6	2.98 149	187 96 91
102	160	44.5 43.0	6	2.98 140	234 88 146
105	176	48.0 42.0	6	2.98 140	228 96 132
117	184	49.5 41.5	6	2.98 144	235 88 147
119	194	47.5 42.5	6	2.98 140	218 100 118
134	168	46.5 43.0	6	2.98 149	179 60 119
137	162	41.0 39.5	6	2.98 144	260 102 158
139	160	43.0 44.5	6	2.98 140	221 100 121
Average	169.7	45.4 40.7			226.3 89.9 136.4

Table 15. Rats selected at random based on pre-injection hematocrit values employed in the assay of erythropoietin as measured by Fe^{59} uptake.

Experiment	Group ¹	Hematocrit ² (average)	Source material	CPM (actual)	Mean % count	Excess over control
1	I	45.2	2	126.2+6.5	108.8	8.8
	II	45.1	1	116.0+6.2	100.0	--
2	III	44.8	3	156.4+7.2	115.4	15.4
	IV	44.9	1	135.5+6.7	100.0	--
3	V	44.9	4	197.9+8.1	116.4	16.4
	VI	45.1	1	170.1+7.5	100.0	--
4	VII	44.2	5	236.8+8.9	131.1	31.1
	VIII	44.4	1	180.6+7.7	100.0	--
5	IX	44.0	6	236.6+8.9	152.0	52.0
	X	44.4	1	155.7+7.2	100.0	--
6	XI	45.6	7	192.1+8.0	140.9	40.9
	XII	45.4	1	156.4+6.8	100.0	--

¹ Ten animals in each group. All source material injected intraperitoneally and all $\text{Fe}^{59}\text{Cl}_3$ injected intracardially.

² Pre-injection hematocrit counts of rats.

In the six experiments with 10 animals assigned to each group, Groups I and II; Groups III and IV; Groups V and VI; Groups VII and VIII; Groups IX and X; and Groups XI and XII, the even-numbered groups were the control animals for each experiment, which received injections of source material (bleeding #1) and the odd-numbered groups were the animals receiving the source material of the other six bleedings (bleedings #2-7, inclusive) listed against their respective controls; that is, number one of the cattle. The average hematocrit values represent random

grouping of the rats by their pre-injection hematocrit values, with a range of 44.0 to 45.6 per cent.

The average actual counts per minute (C/M) for each group of the experiments represents the average values for the 10 animals following subtraction of the background count. The mean percentage count per minute ($\% \bar{C}$) is listed for each of the experimental groups. The values express the effects of bleeding of the cattle; that is, potency of the erythropoietin, and were obtained by expressing each rat's net count per minute as a percentage of the mean net count per minute for the corresponding control group. For example, with rat Group I, rat #X, bleeding #2, the mean net count per minute for the corresponding control rats was 116.0; since rat #X for the treated group with source material, bleeding #2, had a net count per minute of 124; therefore, 124 divided by 116 equals 106.9 as a measure of the #X rat's response to treatment. This was calculated for each rat in Group I and the value of 108.8 expresses the mean count per minute ($\% \bar{C}$) of the 10 rats in Group I. Thus, bleeding #2 exceeded the controls by 8.8 per cent.

The percentage $\text{Fe}^{59}\text{Cl}_3$ uptake obtained in the succeeding experiments was processed in a similar manner: in Experiment 2, bleeding #3 had an increase of 15.4 per cent over their respective controls; Experiment 3, bleeding #4 had an increase of 16.4 per cent over their respective controls; Experiment 4, bleeding #5 had an increase of 31.1 per cent over their respective controls; Experiment 5, bleeding #6 had an increase of 52.0 per

cent over their respective controls; and in Experiment 6, bleeding #7 had an increase of 40.9 per cent over their respective controls. There was a decrease between the sixth and the seventh bleeding of Experiments 5 and 6, but still far above the first bleeding. It will be noted that the actual count per minute of the control groups of rats varied. This could be expected since dealing with different groups of rats.

The reason for the sixth bleeding's response to the erythropoietic stimulation decreasing can not be fully explained. The decreasing response could be due to stressor's effect on the bled bovine animal or overdistension of the cattle's erythropoietic mechanism. The variance in the different rats could account for the varied count per minute between the different rat groups. However, in all instances the data show the trend with bleeding of the cattle; that is, potency of erythropoietin was increased with each bleeding except for the decrease between the fifth and the sixth experiments.

The effects of the bleedings and differential treatment were studied by expressing each rat's "Net C/M" (Net Count per Minute) as a percentage of the mean Net C/M for the corresponding control group. When all six experiments had been handled similarly and the data symbolized by $\% \bar{C}$, the following analysis of variance was obtained:

Source of variation	D/F	Mean squares and significance	
Treatment and bleeding	5	2,820.3	0.1% level
Linear with bleeding	1	11,581.0	0.1% level
Other	4	Maximum mean square - non-significant	
Rats, same treatment	<u>54</u>	793.2	
Total	59		

Figure 1 delineates the results of an analysis of variance and the trend of potency of erythropoietin with bleedings accompanying means count per minute (C/M) for the treated and control groups, and the count per minute (C/M) adjusted for controls against the treated animals in each of the six experiments. Although the controls and treated unadjusted for controls were quadratic trends, after adjustment for the behavior of the controls, the trend with bleeding was predominantly linear as shown by both Fig. 1 and the more detailed analysis of variance, which was statistically significant at the 0.1 per cent level.

The following chart presents the mean % \bar{C} (adjusted count per minute) for each treatment, and also % $\bar{C} - 100$, which was the average amount by which the treated groups exceeded their controls.

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
% \bar{C}	108.8	115.4	116.4	131.1	152.0	140.8
Excess over control	8.8	15.4	16.4	31.1	52.0	40.8

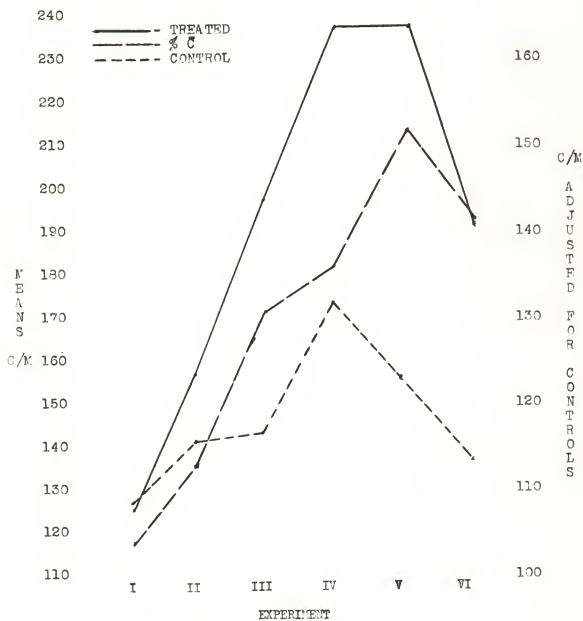


Figure 1. Analysis of Variance Showing the Trend of Potency of Erythropoietin with Bleedings.

The bleedings did not continue long enough to describe if a true quadratic trend or curve was developing as the C/M and $\% \bar{C}$ fall off between the sixth and seventh bleedings.

The Relationship of Hematocrit Values to Erythropoietin

Hematocrit Values of Rats. A correlation between the pre-hematocrit and post-hematocrit values of the rats was obtained, as it might be related to the $\% \bar{C}$ of the rat. A linear correlation coefficient was computed with the following results.

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
Correlation	.05 ns	.46 ns	-.23 ns	.81 *	-.06 ns	-.40 ns

The fifth bleeding was statistically significant at the 1 per cent level; the others were not significant. There was no trend of correlation with bleeding. Hence, it was concluded that there was no discernible relation between changes in hematocrit and $\% \bar{C}$; that is, the adjusted C/M in the rats.

Hematocrit Values of the Cattle. The mean hematocrit values of the cattle at each bleeding also were studied in relation to adjusted C/M($\% \bar{C}$). The correlations of the cattle hematocrit values and the treatment means are presented in the following chart.

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
Mean Ht.	36.43	32.00	28.78	27.25	23.25	22.20
Mean % \bar{C}	108.8	115.4	116.4	131.1	152.0	140.8

The coefficient of simple linear correlation was $-.91$, which was statistically significant at the 1 per cent level. Thus, the increase in adjusted C/M was, aside from the decrease in potency of erythropoietin at the seventh bleeding, very closely associated with the decrease in the hematocrit values of the cattle.

The Relationship of Hemoglobin Values to Erythropoietin

The mean hemoglobin values of the cattle at each bleeding also were obtained for a statistical analysis in relation to adjusted C/M. These hemoglobin values and treatment means are shown in the following chart.

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
Mean Hb.	9.85	8.58	8.15	6.98	6.20	5.99
Mean % \bar{C}	108.8	115.4	116.4	131.1	152.0	140.8

The coefficient of simple linear correlation was $-.94$, which was statistically significant at the 1 per cent level. This suggests that the increase in adjusted C/M was, aside from the decrease of potency of erythropoietin in the seventh bleeding, also very closely associated with the decrease in the cattle hemoglobin values.

The Relationship of Red Blood Cells to Erythropoietin

The mean red blood cell values of the cattle at each bleeding were obtained to substantiate a relationship to adjusted C/M. The correlations of the cattle red blood cell values and the treatment means are presented in the chart below.

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
Mean RBC (in 1000's)	6393	5493	4882	4694	4363	4148
Mean % \bar{C}	108.8	115.8	116.4	131.1	152.0	140.8

The coefficient of simple linear correlation was $-.85$, which was significant at the 10 per cent level. Though significant at a lower level than that for hematocrit and hemoglobin values, it still was indicative of a definite tendency for red blood cells to decrease as mean % \bar{C} increases with each additional bleeding of the cattle.

In general, about the only feature of the changes in C/M adjusted for controls that was not closely associated with such characteristics of the blood studies as hematocrit, hemoglobin, and red blood cell values was the decrease in potency of erythropoietin in % \bar{C} between the sixth and seventh bleedings (fifth and sixth experiment).

The following summary chart which is delineated in Fig. 2 illustrates the above points.

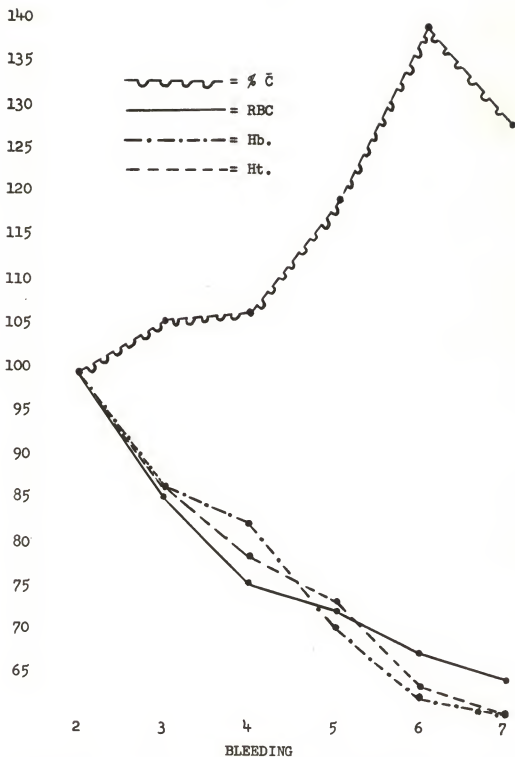


Figure 2. Hemogram of Cattle at Each Bleeding in Relation to Adjusted Count Per Minute ($\% C$).

	Bleeding					
	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
% \bar{C} *	100	106	107	120	140	129
Ht. *	100	87	79	74	64	61
Hb. *	100	87	83	71	63	61
RBC *	100	86	76	73	68	65

* Expressed as percentage reading at bleeding No. 2.

DISCUSSION

Other investigators have presented data to the effect that hypophysectomized, phenylhydrazine treated and starved rats appeared to be more sensitive to the erythropoietic stimulation factor than normal rats (11, 69, 70, 74, 75, 76, 105, 106, 109). However, these data also show that the former elicit an exaggerated response (96) over that of the normal virgin female rats. The sensitivity of the hypophysectomized rat may be due rather to a changed metabolism due to many factors known and unknown, present or absent in the animal with an ablated organ. Other treatments such as injections of phenylhydrazine, or starvation likewise produce profound changes in metabolism. So, although such rats may be more sensitive to erythropoietin and/or other manipulations, the results of erythropoiesis in such animals are exaggerated. Therefore, in these experiments, normal virgin female rats on a balanced regimen containing all the known nutrients were employed as test animals.

The evidence presented in this study has proved that an abundant potent source of erythropoietin may be obtained from cattle blood. The statistical analysis demonstrated the sixth

bleeding was significant at the 0.1 per cent level, although the decreasing of the seventh bleeding in erythropoietin response can not be fully explained at this time. However, in all instances the data present the trend with bleeding the cattle; that is, the potency of erythropoietin was increased between the fifth and sixth experiments. An abundant source of erythropoietin obtained from anemiated cattle induced by hemorrhage can be produced from the immense blood volume of cattle.

The technique of utilizing $\text{Fe}^{59}\text{Cl}_3$ incorporation into the newly-formed erythron as an assay basis was proven. The statistically significant increase in each successive bleeding over its control rats, even the increase over the preceding bleeding, except for the decrease between the sixth and seventh bleedings, was significant. Several investigators (40, 54, 65, 66, 67, 68, 83, 88, 116, 118) have indicated that radioactive iron was a good technique to test for erythropoietin. Recent workers (54, 167) stated that Fe^{59} incorporation probably was the most reliable single method for studying the effect of anemic plasma on erythropoiesis. The average erythrocyte contains approximately 0.1 per cent iron, or about one thousand million atoms of iron in each individual red blood cell. Hahn, et al. (85) stated that radioactive iron was incorporated into the red blood cell as an integral part of the hemoglobin molecule, so if some of the iron atoms involved in this heme synthesis are radioactive, a proportionate number will be incorporated as an integral part of the new hemoglobin molecule in the newly-formed erythron.

The relationship of hematocrit values to erythropoietin, involving the pre-hematocrit or post-hematocrit values of the assay animals, showed no discernible changes in the hematocrits and the adjusted count per minute. Although one coefficient was statistically significant, the others were not, and there was no trend with the bleedings. The mean hematocrits of the cattle bleedings were significant at the 1 per cent level, thus the increase in adjusted count per minute was, aside from the decrease in the sixth experiment, very closely associated with the decrease in the hematocrit values of the cattle.

The effects of hemoglobin values on cattle erythropoietin were significant statistically at the 1 per cent level, and the suggested increase in adjusted count per minute was, aside from the sixth experiment (seventh bleeding) as for hematocrit values, very closely associated with the decrease in the cattle hemoglobin values.

The relationship of red blood cells on cattle erythropoietin was significant at the 10 per cent level, although at a lower level was still indicative of a definite tendency for red blood cells to decrease as count per minute increases with additional bleedings. So, in general, the only feature of the changes in count per minute adjusted for controls not closely associated with such characteristics of the blood, was the hematocrit, hemoglobin, and red blood cell values which decreased in count per minute between the sixth and seventh bleedings (fifth and sixth experiments).

Blood obtained from experimentally induced anemic cattle showed a significant potent cross-species erythropoietic response in standardized normal female rats. This finding is of increasing significance since it may provide an unlimited source for future use of erythropoietin in the study of various types of anemia, irradiation injury, and disturbances of the blood-forming organs. The role of erythropoietin in relation to the incorporation of iron into cells other than those of the erythron presents itself for further vista of investigation. An abundant source of erythropoietin has not been reported previously. Since this study demonstrated marked potency following a year of storage, little is left to be desired for an abundant source of erythropoietin. Whether erythropoietin is a universal substance present in all species of mammals and identical in chemical and physical characteristics, remains to be elucidated. Further investigations utilizing exsanguination in combination with phenylhydrazine treatment may produce a quicker and more profound source of erythropoietin in the different mammalian species.

In summary, the following chart delineates the different factors affecting erythropoiesis or dynamic equilibrium of the erythron.

<u>Conditions</u>	<u>Effects</u>
¹ <Demand for oxygen while supply is normal (Starvation, hypophysectomy).	<Erythropoiesis ²
>Supply of oxygen while demand is normal (Transfusion-induced polycythemia, hyperoxia).	<Erythropoiesis ²
>Demand for oxygen while supply is normal (Dinitrophenol, triiodothyronine).	>Erythropoiesis
<Supply of oxygen while demand is normal (Bleeding, phenylhydrazine-induced hemolysis). ³	>Erythropoiesis

¹ >,< is increase or decrease, respectively.

² Exaggerated response to erythropoietin (anemic plasma).

³ Standard procedures to obtain plasma with high erythropoietic activity in rats.

SUMMARY

Twelve yearling Hereford heifers and 120 virgin female Sprague-Dawley rats were utilized in six experiments to demonstrate whether an erythropoietic factor(s) was present in bovine plasma, to assay the potency of bovine erythropoietin, and to determine its cross-species potency and possible uses following an extended period of storage. The results of this study proved that cattle blood contained an erythropoietic factor(s) as elucidated by statistical analysis, which was significant at the 0.1 per cent level, although the seventh bleeding (sixth experiment) decreased in erythropoietic activity. However, in all instances, data present the trend with bleeding the cattle; that is, the

the potency of erythropoietin was increased, ^{except} between the sixth and seventh bleedings. Cattle blood can elicit an abundant source of erythropoietin due to the immense blood volume of anemiated cattle.

The technique of $\text{Fe}^{59}\text{Cl}_3$ incorporation into newly-formed erythrons was proved, as the increase in the bleedings over its control group of rats was statistically significant. The potency of the cattle erythropoietic factor(s) demonstrated a significant response in cross-species relationship in the rats as the recipient animals. The bovine erythropoietin, after a year in storage at 5°C ., was still significantly potent.

The relation of pre-hematocrit and post-hematocrit values of the assay animals showed no discernible changes in the hematocrits and the adjusted count per minute. The cattle mean hematocrit values were statistically significant at the 1 per cent level and very closely associated with the decrease in the cattle hematocrits. The hemoglobin values of the cattle also indicated an effect on erythropoietin with significance at the 1 per cent level, which was very closely associated with the decrease in the cattle hemoglobin values.

The relationship of red blood cells on cattle erythropoietin was statistically significant at the 10 per cent level, although at a lower level it was still indicative of a definite tendency for erythrocytes to decrease as counts per minute increase with additional bleedings. In general, the only feature of the changes in count per minute adjusted for controls; that is, not

closely associated with such characteristics of the blood as was the hematocrit, hemoglobin, and red blood cell values, was the decrease in count per minute between the sixth and the seventh bleedings.

It is postulated that cattle blood (partial protein-free plasma) contains and elicits a potent erythropoietic factor(s) that can be utilized in cross-species relationships, which is significantly active after storage at 5° C. for almost one year. The $\text{Fe}^{59}\text{Cl}_3$ technique is one of the best methods to demonstrate the erythropoietic potency of cattle plasma. The potential source of erythropoietin in the bovine animal is abundant, due to the enormous blood volume. Further investigations are needed to fulfill the much sought after knowledge in this field of erythropoiesis.

EXPLANATION OF PLATE I

Erythropoietin preparations

- Fig. 1. Pooled cattle erythropoietin sample; partial protein-free filtrate preparation.
- Fig. 2. Sheep erythropoietin; desiccated sample of PPF obtained from phenylhydrazine-treated animals. (Courtesy of Armour and Company.)

PLATE I



Fig. 1

Fig. 2

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EVALUATION OF AN ERYTHROPOIETIC FACTOR OBTAINED
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CATTLE USING RADIOACTIVE Fe^{59}

by

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The erythropoietic stimulating activity of the serum of rabbits made anemic by bleeding created little interest for some 40 years, but has received a great deal of recognition, especially since 1950 and including the present time. The term "erythropoietin" was used to designate the factor(s) found in plasma of animals made anemic by bleeding or by other methods which accelerate erythropoiesis when such plasma is injected into assay animals. The name first given to this substance which stimulated red blood cell formation was "hemopoietine." Although the plasma factor's chemical nature and mode of action still are not completely understood, some of its physical and chemical properties have been described.

The experiments were designed to demonstrate whether an erythropoietic factor(s) was present in bovine plasma, determine the cross-species potency and the effect of extended periods of storage, and to assay the potency of the erythropoietin of cattle blood. The procedures were designed also to demonstrate an abundant supply source of erythropoietin and its relationship upon the hematocrit, hemoglobin, and red blood cell values during the exsanguinations.

The technique utilized normal Hereford yearling heifers as donors, which were bled at weekly intervals until their hematocrit values decreased below one-half their normal value. Rats (virgin females of the Sprague-Dawley strain) were employed for the assay which was designed for statistical analysis. The experiments were delineated to further the knowledge concerning the

erythropoietic stimulating substance.

Radioactive iron⁵⁹ was employed because it is incorporated immediately and preferably to storage iron in the process of erythropoiesis and serves as a useful element in this respect. The average actual counts per minute for each group for each experiment represent the average values of the animals following subtraction of the background count. The mean percentage count per minute values express the effects of bleeding of the cattle; that is, potency of erythropoietin, and were obtained by expressing each rat's net count per minute as a percentage of the mean net count per minute for the corresponding control groups.

It was noted that the actual count per minute of the control group of rats varied. This could be expected since different groups of rats were utilized in the experiments. However, in all instances, the data showed the trend with bleeding of the cattle; that is, potency of erythropoietin was increased with bleedings except for a decrease between the sixth and seventh bleedings. The treated and the control animals showed quadratic trends, but after adjusting the behavior of the controls, the trend with bleeding was predominantly linear and was statistically significant at the 0.1 per cent level.

The mean hematocrit, hemoglobin, and red blood cell values of the bovine animals at each bleeding were studied in relation to the adjusted count per minute. The increase in the adjusted count per minute was, aside from the decrease in the seventh bleeding, very closely associated with the decrease in the

hematocrit, hemoglobin, and red blood cell values. Each of the above demonstrated a statistically significant increase in each experiment except for the percentage decrease between the sixth and seventh bleedings.

In the six experiments, the incorporation of $\text{Fe}^{59}\text{Cl}_3$ into the newly-formed erythrocyte was utilized as an index of red blood cell formation. It was proven that partial protein-free filtrate obtained from cattle made anemic by bleeding contained a potent erythropoietic factor(s) as shown by the rat assay technique. Thus, there is an abundant source of erythropoietin in the blood of cattle because of their large blood supply. The source material showed marked potency following a year of storage. Further investigations are needed in the field of erythropoiesis to increase our knowledge on the stimulating activity of the erythropoietic factor present in cattle blood.